Lady-slipper orchid mycorrhizal associations reveal specificity suggestive of resource fragmentation and resource tracking

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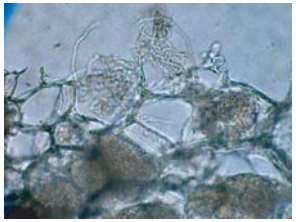
Fig. 1: *Cypripedium calceolus*

Fig. 2: *Cypripedium fasciculatum*

Fig. 3 (above): Cross-section of mycorrhizally colonized *C. guttatum root*. **Fig 4** (below): Close-up of peloton, or mycorrhizal hyphal coil, in *C. guttatum*



Introduction

Terrestrial lady's slipper orchids in genus *Cypripedium* grow throughout the temperate Northern Hemisphere. Though many are geographically widespread, all are locally rare. Considered among the most beautiful of orchids, they are protected throughout the world through international treaties and conservation laws. The rarity of *Cypripedium* has attracted study into conservation via cultivation and habitat management. Identification of the mycorrhizal fungi is imperative for both efforts. *Cypripedium* spp. have been the object of mycorrhizal study for over 150 years, but results have been inconclusive.

We used modern DNA methods to assess the mycorrhizal symbionts of seven *Cypripedium* species occurring in North America and Europe: *C. calceolus* (Fig. 1), *C. californicum*, *C. candidum*, *C. fasciculatum* (Fig. 2), *C. guttatum*, *C. montanum*, and *C. parviflorum*. The first stage of this work, dealing with family-level classification of mycorrhizal fungi via analysis of fungal nucLSU and mtLSU rDNA, has been published (Shefferson et al. 2005. *Mol Ecol* 14: 613-626). Here, we present new analyses conducted with unpublished ITS sequence data.

Methods

Several plants were sampled from multiple populations per species. Because most populations were small, samples represent large proportions of their populations. Roots were systematically examined for pelotons (Fig. 3 & 4), the primary evidence of mycorrhizal colonization in orchids. Bulk DNA was extracted from colonized root sections.

We first PCR amplified the fungal ITS-nucLSU DNA with several primer pairs and performed RFLPs of all root section samples. Representative RFLP types from each plant were sequenced. Samples with RFLP patterns suggesting multiple fungi were PCR-cloned, and the clones sequenced. Representative samples were chosen for nucLSU and mtLSU sequencing, and phylogenetic analysis with reference databases of sequences suggested the dominant mycorrhizal association to be with family Tulasnellaceae (Fig. 5), although Ceratobasidiaceae and Sebacinaceae were also found.

Here, we analyze ITS data via ML under model HKY using MetaPIGA. 250 replicates of 4 populations with 4 trees each were used.

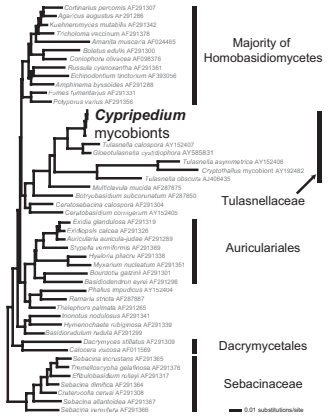


Fig. 5: Nuclear large subunit rDNA phylogeny of the Hymenomycetes, showing the phylogenetic position of the dominant clade of *Cypripedium* mycobionts.

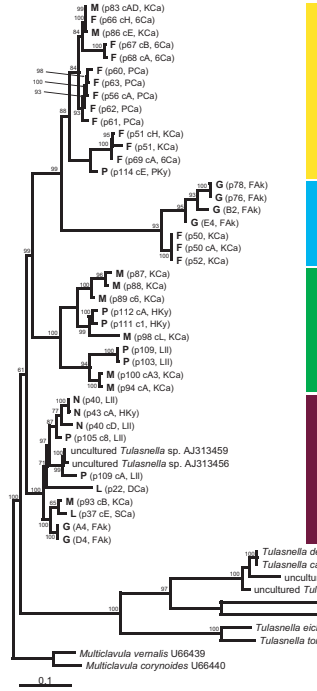


Fig. 6 (left): ITS phylogeny of *Cypripedium* mycobionts occurring in family Tulasnellaceae. Phylogeny constructed with 740bp alignment, and rooted with *Multiclavula vernalis* and *M. corynoides*. Heuristic ML analysis with bootstrap derived from ML replicates in MetaPIGA. Species key in Results above.

Results: Across Species

Four tulasnelloid clades were supported via ML analysis, all forming one monophyletic group corresponding to or sister to genus *Tulasnella*. Species-by-species, the results are as follows:

Cypripedium calceolus (**C**): So far, no ITS has been successfully PCR amplified. Only mtLSU amplification has been successful. Potentially a different group of tulasnelloids.

C. californicum (**L**): Limited to clade 1.

C. candidum (**N**): Limited to clade 1, even across populations ~1000km apart.

C. fasciculatum (**F**): Limited to clades 3 and 4.

C. guttatum (**G**): Limited to clades 1 and 3.

C. montanum (**M**): Wide specificity within family Tulasnellaceae, including clades 1, 2, and 4.

C. parviflorum (**P**): Wide specificity within family Tulasnellaceae, including clades 1, 2, and 4.

Results: Geographic Variation

C. californicum: Associated with tulasnelloid clade 1 and family Sebacinaceae in central coastal California, but with tulasnelloid clade 1 and the Ceratobasidiaceae in far northern California (Fig. 7). Associations between may be with *Glomus*.

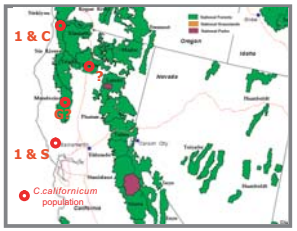
C. candidum: No variation observed. All populations associated with a tight clade within tulasnelloid clade 1 (Fig. 6).

C. fasciculatum: Associations were exclusively with tulasnelloid clade 4 in the northern Sierra Nevada and in Six Rivers National Forest, but also included clade 3 near the Oregon border (Fig. 8). In Mendocino National Forest, associations were strictly with the Sebacinaceae. Mycorrhizal fungal identity could not be determined for populations near Mt. Shasta.

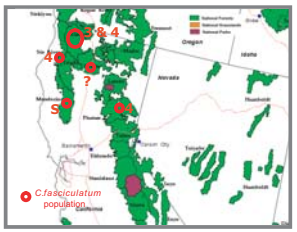
C. montanum: Populations in Klamath National Forest associated with tulasnelloid clades 1, 2, and 4, irrespecive of population.

C. parviflorum: A n. Illinois population associated with tulasnelloid clades 1 and 2. One Kentucky population associated with clade 2 only, while another associated with clade 4 and family Sebacinaceae.

Limited sampling has prevented geographic variation in *C. calceolus* and *C. guttatum* mycorrhizae from being ascertained.



Maps of *C. californicum* (above, **Fig. 7**) and *C. fasciculatum* (below, **Fig. 8**) sampled regions in n. California. Key: Mycorrhizal association with (1-4) Tulasnellaceae in ITS clades 1-4, respectively; (S) Sebacinaceae; (C) Ceratobasidiaceae; (G?) *Glomus* spp. (uncertain); and (?) uncertain.



Discussion: Mycorrhizal Evolution in *Cypripedium* and the Orchidaceae

Fungi in families Tulasnellaceae and Ceratobasidiaceae appear to be the mycorrhizal partners of the most basal branches of the monophylletic family Orchidaceae, including the Apostasioideae and Cypripedioideae. However, while Tulasnellaceae remains the dominant mycorrhizal partner of *Cypripedium* spp., association with Ceratobasidiaceae is only visible in the most ancestral species of *Cypripedium* - *C. californicum*. Associations with family Sebacinaceae also occur in *C. californicum*, as well as in *C. fasciculatum* and *C. parviflorum*, but are unknown to occur in more ancestral branches of the Orchidaceae. We suggest that associations with Ceratobasidiaceae were most likely lost after the speciation event resulting in *C. californicum*, while associations with Sebacinaceae most likely evolved at least twice in the genus.

Within family Tulasnellaceae, association with clade 1 appears ancestral. Association with clade 2 may have evolved only once - after the branching event leading to the *C. guttatum*/*C. fasciculatum* clade, and prior to the speciation event leading to *C. montanum*. This association may have been lost in *C. candidum*. Association with clade 3 appears only in the *C. guttatum*/*C. fasciculatum* clade, suggesting it evolved once. Association with clade 4 occurs in *C. fasciculatum*, *C. parviflorum*, and *C. montanum*, but has so far never been observed in other species, suggesting several gains and/or losses of this association.

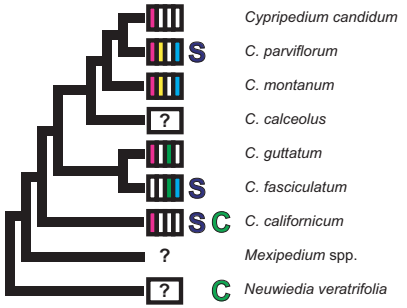


Fig. 9: Phylogenetic tree of sampled *Cypripedium* species, based on 5s rDNA tree from Cox (1995). Rooted with *Neuwiedia*, in the Apostasioideae. Mycorrhizal associations shown as:

Discussion: Coevolution?

At the fungal family level, *Cypripedium* spp. appear highly specialized to their mycorrhizal hosts. At finer scales, these interactions seem more dynamic. But because they are rare and not likely to be the primary mycorrhizal partners of their fungal hosts, and because they are not likely to benefit their mycorrhizal fungi, *Cypripedium* spp. cannot be said to undergo mycorrhizal coevolution. Instead, mycorrhizal variation among both species and populations suggests **resource tracking** - *Cypripedium* spp. evolutionarily track a resource provided by multiple partners, and shift among them without creating an adaptive response in the fungus. Mycorrhizal evolution in genus *Cypripedium* may also be an example of **resource fragmentation** - specialization may be dependent on the frequency of contact with different mycorrhizal fungi. Thus, the rarest *Cypripedium* species, *C. californicum*, should be the least specialized due to limited contact.

Fig. 11: Pelotons in *Cypripedium japonicum*.

Fig. 10: Seeds of *Cypripedium montanum*, which have been planted in northern California to examine mycorrhizal colonization of seeds and seedlings.

Further Work

1. Now finishing a project on *in situ* seed germination to assess seedling mycorrhizal associations (Fig. 10).

2. Now sampling most *Cypripedium* spp., analyzing both mycorrhizal and plant phylogenies (Fig. 11).

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